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# Estrogen Related Receptor, $ERR\alpha$ , A Regulator of Bone Formation

## Field of the Invention

The present invention relates to the orphan estrogen related receptor,  $ERR\alpha$ , which is involved in the regulation of bone formation

## Background of the Invention

Nuclear receptors are transcription factors involved in various physiological regulatory processes. The superfamily to which nuclear receptors belong comprises both ligand-dependent molecules such as the steroid hormone-, thyroid hormone-, retinoic acid- and vitamin D-receptors, and an increasing number of so-called orphan receptors for which no ligand has yet been determined (Gronemeyer H, Laudet V., 1995, Enmark and Gustafsson, 1996). Indeed, it is not yet known whether the orphan receptors have ligands that await identification or whether they act in a constitutive manner. The orphan receptors display the same structural organization as do the classic ligand-dependent receptors: the A/B domain located in the N-terminal part of the protein harbors a ligand-independent transactivation function (AF-1), the C domain, which is the most conserved part of the molecule, is responsible for the specific DNA-binding activity; the E domain contains the ligand binding hydrophobic pocket and contributes to receptor dimerization and to the ligand-dependent transactivation function (AF-2).

Two orphan receptors, estrogen-related receptor  $\alpha$  ( $ERR\alpha$ ) and  $ERR\beta$  (Giguere et al., 1988; NR3B1 and NR3B2, respectively, according to the Nuclear Receptors Nomenclature Committee, 1999) are closely related to the estrogen receptors  $ER\alpha$  and  $ER\beta$  (Green et al., 1986; Kuiper et al., 1996; NR3A1 and NR3A2 respectively).  $ERR\alpha$  and  $ERR\beta$  were identified by low-stringency screening of cDNA libraries with a probe encompassing the DNA-binding domain of the human estrogen receptor (ER). Sequence alignment of  $ERR\alpha$  and the ERs reveals a high similarity (68%) in the 66 amino acids of the DNA-binding domain and a moderate similarity (36%) in the ligand-binding E

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domain, which may explain the fact that  $ERR\alpha$  does not bind estrogen.  $ERR\alpha$  has been identified as a regulator of the SV40 major late promoter during the early-to-late switch of expression (Wiley et al., 1993) and as a regulator of fat metabolism (Sladek et al., 1997). Yang et al. also showed that  $ERR\alpha$  modulates the activating effect of estrogens on the lactoferrin promoter and suggested that  $ERR\alpha$  may interact with ERs through protein-protein interaction (Yang et al., 1996).

An anabolic effect of estrogens on bone homeostasis has been documented in postmenopausal osteoporosis (for review see Pacifi, 1996), where bone loss can be retarded reversed by administration of natural or synthetic estrogens. However, the molecular mechanisms by which these compounds act is still poorly understood, as is the nature of their cellular target(s). Bone is a tissue undergoing constant renewal, a remodelling process controlled by osteoblasts that form bone and osteoclasts that resorb it. Although the ERs are expressed at relatively low levels in osteoblasts and even lower levels in osteoclasts (Turner et al., 1994), estrogens have been found to elicit effects ranging from modulation of gene expression to regulation of proliferation in both cell types. Due to their homology to the ERs, we hypothesized that the ERRs may intervene in the signals induced by estrogen in bone.  $ERR\beta$  expression, however, is restricted to early development and to a few adult tissues (Giguere et al., 1988; Pettersson et al., 1996). In contrast,  $ERR\alpha$  has a broader spectrum of expression, including fat, muscle, brain, testis and skin (Bonnelye et al., 1997b). Strikingly,  $ERR\alpha$  is also highly expressed in the ossification zones of the mouse embryo (in long bones, vertebrae, ribs and skull), and is more widely distributed in osteoblast-like cells than is  $ER\alpha$  (Bonnelye et al., 1997a). Moreover it has been shown that  $ERR\alpha$  positively regulates the osteopontin gene (Vanacker et al., 1998), an extracellular matrix molecule secreted by osteoblasts and thought to play a role in bone remodelling (Denhardt and Guo, 1993), especially resorption (ref).

Given these observations, we sought to assess more directly whether  $ERR\alpha$  plays a functional role in bone formation. The present invention demonstrates that  $ERR\alpha$  is more highly expressed in differentiating osteoblastic cells than either  $ER\alpha$  and  $ER\beta$  and that it is differentially regulated during the developmental sequence. The present invention also demonstrates that upregulation of  $ERR\alpha$  levels stimulates bone formation while downregulation inhibits bone formation. Our data suggest that modulation of  $ERR\alpha$  levels may be a useful therapeutic tool in diseases of bone metabolism.

### Summary of the Invention

The present invention is directed to the orphan estrogen related receptor,  $ERR\alpha$ , expressed by osteoblastic cells which is a potentially important regulator of bone formation and maintenance of bone mass. It has been found that  $ERR\alpha$  is differentially expressed throughout all osteoblastic differentiation stages *in vitro*.  $ERR\alpha$  is much more highly expressed in osteoblastic cells than either  $ER\alpha$  or  $ER\beta$ .  $ERR\alpha$  is expressed more highly in cuboidal osteoblastic cells than in surrounding non-nodular/fibroblastic cells and nuclear expression of  $ERR\alpha$  increased as osteoblasts matured. Immunocytochemistry also showed that  $ERR\alpha$  and  $ER\beta$  but not  $ER\alpha$  are highly expressed in the developing 21d fetal rat calvaria in sutural cells and cells at the osteogenic front. On the other hand, little  $ER\beta$ , but abundant  $ERR\alpha$  and  $ER\alpha$ , are expressed in mature osteoblasts and osteocytes in compact and remodeling bone.

Blocking  $ERR\alpha$  expression in either proliferating or differentiating RC cell cultures was found to inhibit cell growth and also demonstrated a proliferation-independent complete inhibition of both mineralized and unmineralized bone nodule formation. Overexpression of  $ERR\alpha$  with transient transfection of RC cells resulted in a dose dependent biphasic effect.

Taken together, the findings show that  $ERR\alpha$  is more highly expressed in osteoblastic cells than either  $ER\alpha$  or  $ER\beta$ , that  $ERR\alpha$  is differentially regulated during the developmental sequence, and that the three receptors are differentially expressed in different cohorts of osteoblasts.

It is therefore apparent that  $ERR\alpha$  plays a physiological role in bone formation at both proliferation and differentiation stages. This indicates a therapeutic role for this nuclear transcription factor in various clinical conditions such as osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, periodontal disease, tumoral calcinosis and dermatomyositis.

According to an aspect of the present invention is the use of  $ERR\alpha$  in the regulation of bone formation in a mammal.

According to another aspect of the present invention is the use of the  $ERR\alpha$  for the treatment of diseases of bone metabolism.

According to a further aspect of the present invention is the use of  $ERR\alpha$  for the treatment of a condition selected from the group consisting of osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, periodontal disease, tumoral calcinosis and dermatomyositis.

According to yet a further aspect of the present invention is the use of  $ERR\alpha$  for the regulation of bone formation during fetal development and also in adult mammals.

According to still a further aspect of the present invention is the use of  $ERR\alpha$  for stimulating the differentiation of osteoblasts from primitive progenitor cells to mature osteoblasts and osteocytes *in vitro* and *in vivo*.

The present invention also provides a therapeutic composition, comprising a purified  $ERR\alpha$  as active ingredient, and a method for the treatment of various clinical conditions involving abnormal bone formation. In particular, the composition and method can be used to treat osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, periodontal disease, tumoral calcinosis and dermatomyositis.

In accordance with one aspect of the invention there is provided a method for the treatment of a bone developmental disorder in a mammal comprising administering to a mammal an effective amount of a composition which comprises as an active ingredient  $ERR\alpha$  in a mixture with a pharmaceutically acceptable diluent or carrier. It is also acknowledged that the  $ERR\alpha$  receptor can also be used to regenerate tissues after tissue damage, namely in bone.

In accordance with a further embodiment, the  $ERR\alpha$  signaling pathway may be modulated by modulating the binding of the  $ERR\alpha$  to an  $ERR\alpha$  binding partner. Such a binding partner may include for example the estrogen receptor.  $ERR\alpha$  can be used to upregulate the transcription and thus expression of certain genes which work together with  $ERR\alpha$  to affect skeletal development.

The invention further provides methods for screening candidate compounds to identify those able to modulate signaling by  $ERR\alpha$  through a pathway involving  $ERR\alpha$ .

For example, the invention provides screening methods for compounds able to bind to  $ERR\alpha$  which are therefore candidates for modifying the activity of  $ERR\alpha$ . Various suitable screening methods are known to those in the art, including immobilization of  $ERR\alpha$  on a substrate and exposure of the bound  $ERR\alpha$  to candidate compounds, followed by elution of compounds which have bound to the  $ERR\alpha$ .

The invention also provides a method of modulating a  $ERR\alpha$  signaling pathway by increasing or decreasing the availability of  $ERR\alpha$  or by modulating the function of the  $ERR\alpha$ .

The invention further provides methods for preventing or treating diseases characterised by an abnormality in an  $ERR\alpha$  signaling pathway which involves  $ERR\alpha$ , by modulating signaling in the pathway.

In a further embodiment, a normal version of  $ERR\alpha$ , could be provided by gene therapy to restore function in a disease wherein  $ERR\alpha$ , is mutated or non-functional.

According to a further aspect of the present invention, transgenic animal models containing a mutant  $ERR\alpha$  or alternatively have been developed as a  $ERR\alpha$  gene knock out can be used in order to further characterize the role of  $ERR\alpha$  in skeletal development. Such a transgenic system also allows for screening of candidate compounds as therapeutics. For example, knock out animals, such as mice, may be produced with deletion of a  $ERR\alpha$  gene. These animals may be examined for phenotypic changes and used to screen candidate compounds for effectiveness to reverse these changes.

According to a further aspect of the present invention  $ERR\alpha$  expression can be used to target and identify genes activated during particular osteoblast developmental time windows.

In accordance with another aspect of the present invention is a method for screening a candidate compound for effectiveness as an antagonist of  $ERR\alpha$  comprising:

- (a) providing an assay method for determining a biological activity of  $ERR\alpha$ ,  
and
- (b) determining the biological activity of  $ERR\alpha$  in the presence or absence of the candidate compound, wherein a reduced biological activity in the presence of the candidate compound indicates antagonist activity of the compound.

In accordance with another aspect of the present invention is a method for treating in a mammal a disorder associated with an undesired biological activity of  $ERR\alpha$  comprising administering to the mammal an effective amount of a substance selected from the group consisting of

- (a) an  $ERR\alpha$  antagonist,
- (b) an antibody which binds specifically to  $ERR\alpha$ ; and
- (c) an antisense strand comprising a nucleic acid sequence complementary to the sequence or fragment of the sequence and capable of hybridizing to the nucleic acid sequence encoding  $ERR\alpha$ ; and
- (d) an agent which down regulates the expression of the  $ERR\alpha$  gene encoding for  $ERR\alpha$ .

According to another aspect of the present invention is a method for suppressing in a mammal, the proliferation of a cell capable of being stimulated to proliferate by  $ERR\alpha$ , the method comprising administering to the mammal an effective amount of a  $ERR\alpha$  antagonist or an antibody which binds specifically to  $ERR\alpha$ . Such cells would include but not be limited to primitive progenitor cells, osteoblasts and osteocytes.

### Brief Description of the Drawings

The present invention will be further understood from the following description with reference to the Figures, in which:

Figure 1 shows a northern blot in which  $ERR\alpha$  mRNA from rat calvaria cells grown in the presence and absence of dexamethasone is differentially expressed during osteoprogenitor cell differentiation to mature osteoblasts and osteocytes,

Figure 2 shows  $ERR\alpha$  mRNA expression in osteoblast lineage cells at all development times selected on the basis of molecular phenotype of osteopontin, bone sialoprotein, alkaline phosphatase and osteocalcin expression.

Figure 3 shows the expression of  $ERR\alpha$  compared with that of  $ER\alpha$  and  $ER\beta$  in RC cell cultures in the presence and absence of dexamethasone,

Figure 4 shows immunocytochemistry using appropriate antibodies and antiserum of RC cells showing different expression patterns of  $ERR\alpha$ ,  $ER\alpha$  and  $ER\beta$  over time from day 2 to day 15.

Figure 5 shows immunocytochemistry of 21 day fetal rat calvaria sections showing different expression patterns of  $ERR\alpha$ ,  $ER\alpha$  and  $ER\beta$ ;

Figure 6 shows a dose dependent decrease in RC cell number using antisense oligonucleotide specific for  $ERR\alpha$ ;

Figure 7 shows the effect of antisense oligonucleotide treated RC cells from day 6 of cell culture on nodule formation;

Figure 8 shows the overexpression of  $ERR\alpha$  in transiently transfected RC cells on the formation of mineralized bone nodules; and

Figure 9 shows the effect of estrogen and vitamin  $D_3$  on  $ERR\alpha$  levels in RC cell cultures.



### Detailed Description of the Preferred Embodiments

In accordance with the present invention, it has now been demonstrated that  $ERR\alpha$  plays a role in the regulation of bone formation and as such has several potential therapeutic uses especially in the treatment of diseases involving altered bone metabolism.  $ERR\alpha$  has potential therapeutic use in the treatment of various diseases such as but not limited to osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, periodontal disease, tumoral calcinosis and dermatomyositis.

$ERR\alpha$  can be used both *in vitro* and *in vivo* to stimulate proliferation and differentiation of osteoblasts from primitive progenitors to mature osteoblasts and osteocytes. Thus it has therapeutic utility for increasing bone mass in such conditions where bone mass is decreased as for example in osteoporosis.

#### $ERR\alpha$ mRNA is differentially expressed in developing osteoblast lineage cells

It is now demonstrated that  $ERR\alpha$  mRNA is expressed in differentiating primary cultures of rat calvaria cells and in single isolated osteoblast colonies at different stages of differentiation. However, levels were generally lower in primitive progenitors, more mature precursors, and osteoblastic cells associated with mineralized nodules, and higher in preosteoblasts and osteoblasts. These results suggest that  $ERR\alpha$  may have a function in osteoblasts throughout their developmental lifetime, i.e. from primitive progenitor to the mature osteoblasts and osteocytes, and throughout the lifetime of the organism, i.e.,  $ERR\alpha$  protein was detected *in vivo* in 21 day fetal (see also below) and in adult calvaria, where  $ERR\alpha$  is still expressed in osteocytes (data not shown). It has also been demonstrated that  $ERR\alpha$  is expressed throughout osteogenesis in rat bone marrow stromal cell cultures (data not shown). These results suggest that  $ERR\alpha$  may have functions not only during fetal development but also in adult life in both bone and bone marrow stroma. Strikingly,  $ERR\alpha$  mRNA is also more highly expressed than either  $ER\alpha$  or  $ER\beta$  mRNA. Taken together, these data may indicate that  $ERR\alpha$  has a more widespread activity in bone than either  $ER\alpha$  and  $ER\beta$ .

ERR $\alpha$  mRNA is more highly and widely expressed than either ER $\alpha$  and ER $\beta$

ERR $\alpha$  protein was found more widely distributed *in vitro* in RC cell cultures than either ER $\alpha$  or ER $\beta$ . ERR $\alpha$  was found in most if not all cells in RC cell cultures from early proliferation stages through mineralized nodule formation. ER $\alpha$  was also detected in RC cells at all times analysed but at lower levels than ERR $\alpha$  which is also the case in rat bone marrow cultures (data not shown). ER $\beta$ , on the other hand, was more difficult to detect at any time other than in early proliferating cultures. These observations fit with the expression pattern of these three receptors *in vivo* in 21 day fetal calvariae. Indeed, ERR $\alpha$  is more highly and widely expressed than either ER $\alpha$  or ER $\beta$ , being highly expressed in sutural cells and all identifiable osteoblasts and osteocytes. ER $\alpha$  is not highly expressed in nascent, but is detectable in more mature, osteoblastic cells. ER $\beta$ , on the other hand, is more highly expressed in sutural and nascent osteoblasts. These results suggest that ERR $\alpha$  and ER $\alpha$  may be co-expressed in at least some osteoblastic cells, and that these receptors may regulate the expression of the same target genes in bone via their known ability to participate in protein-protein interactions (Johnston et al, 1997) and their recently described capacity to bind to the same DNA target sequence on the osteopontin promoter (Vanacker et al, 1999). Whether ERR $\alpha$  and ER $\beta$  co-expression also occurs in early osteoblastic cells (osteoprogenitors) is a possibility based on the present data but interactions between these two receptors has not yet been described although they do recognize the same ERE response element as recently described (Vanacker et al, 1999). It is also notable that ERR $\alpha$  protein is localized primarily in either the nucleus or the cytoplasm or both depending on the developmental stage of the osteoblast. This suggests that ERR $\alpha$  target genes and function may vary depending on the maturational stage of the osteoblastic cells.

#### ERR $\alpha$ is required for proliferation of RC cells and for the formation of bone nodules

Consistent with its expression in proliferating RC cells, we have found that downregulation of ERR $\alpha$  inhibits proliferation of RC cell populations, an inhibition that may have consequences on bone nodule formation at later times. However, downregulation of ERR $\alpha$  also decreases differentiation and bone nodule formation in a proliferation-independent manner in RC cells cultures. Upregulation of ERR $\alpha$  had biphasic effects on bone formation, either increasing or decreasing bone nodule formation depending on the amount of overexpressed ERR $\alpha$ . These results suggest an essential role for ERR $\alpha$  in proliferation, differentiation and bone nodule formation in osteoblastic populations *in vitro*, predicting an important role for this receptor in the formation and turnover of the skeleton. Bone specific knockout of ERR $\alpha$  or ERR $\alpha$  overexpression experiments in transgenic mice will test these possibilities.

These functions of ERR $\alpha$  correlate with the known function of ER $\alpha$  in bone. For example, the knockout of ER $\alpha$  caused a 20% reduction in bone mass (Korach, 1994). The fact that ERR $\alpha$  and ER $\alpha$  appear to be co-expressed in at least some osteoblast populations, and may have the capacity to functionally interact, suggests that they may have at least some of the same functions in bone, including an activity in bone formation.

#### ERR $\alpha$ expression is stimulated by estrogen and vitamin D3 but not by dexamethasone in proliferating RC cell cultures

ERR $\alpha$  is expressed in RC cell populations and the level of expression is almost the same in cells not treated (-Dex) or treated with dexamethasone (+ Dex). A 24 hour acute treatment of RC cells with Dex at day 9 or day 15 also had no effect on the ERR $\alpha$  expression level. These results suggest that Dex has no effect on the expression of ERR $\alpha$  while it does downregulate ER $\alpha$  and even more potently downregulates ER $\beta$ . These results suggest that the regulation by glucocorticoids of ERR $\alpha$  differs from that of the ERs.

It was also demonstrated that estrogen (E2) regulates  $ERR\alpha$  at early times in chronically treated RC cell cultures, while an acute (24h) treatment at either day 9 or day 15 did not. These results suggest a link between  $ERR\alpha$  and E2 in bone during proliferation. Shigeta et al. showed that E2 can also activate  $ERR\alpha$  in the uterus (Shigeta et al, 1997). Together, these results suggest a link between  $ERR\alpha$  and estrogens in two estrogen-sensitive tissues.

$ERR\alpha$  and  $ER\alpha$  are both expressed in adult osteocytes suggesting a function of  $ERR\alpha$  during adult life and, consequently, may be involved in some diseases characterized by a decrease in bone mass such as osteoporosis. Bone loss in the aging skeleton is accelerated by a decrease in secretion of estrogens in post-menopausal women and can be reversed by administration of natural or synthetic estrogens. The decrease in estrogen also induces a decrease in the expression of  $ER\alpha$  (Braidman et al, 1998). It is possible that  $ERR\alpha$  is also regulated by estrogens in the aging skeleton and may have a role in osteoporosis.

$ERR\alpha$  is also regulated by another hormone involved in bone metabolism, namely  $1,25 (OH)_2 D_3$ , during the proliferation stages of RC cell cultures. As with E2, acute treatment with  $1,25 (OH)_2 D_3$  for 24 hours at day 9 or day 15 had no effect on  $ERR\alpha$  expression. It will be determined whether  $1,25(OH)_2D_3$  effects may be mediated at least in part by its abilities to modify  $ERR\alpha$  levels.

In conclusion,  $ERR\alpha$  is expressed in osteoblastic cells at levels higher than either  $ER\alpha$  or  $ER\beta$ .  $ERR\alpha$  appears to have a function in proliferation, differentiation and bone nodules formation in RC cells. Finally,  $ERR\alpha$  is regulated by E2 and  $1,25 (OH)_2 D_3$ , two important hormones known to have strong effects on bone formation and turnover. All these results appear to converge in the same pathway and suggest that  $ERR\alpha$  may have a important function in the formation and turnover of the skeleton.

Transgenic non-human animal models may be made for the study of the effects of over and under expression of the  $ERR\alpha$  gene, for the screening of candidate compounds as potential antagonists of this receptor and for the evaluation of potential therapeutic interventions.

The transgenic animals of the invention may also provide models of disease conditions associated with abnormalities of  $ERR\alpha$  expression. For example, the transgenic animals of the invention may provide an animal model of at least some aspects of rheumatoid arthritis.

Animal species suitable for use in the animal models of the invention include mice, rats, rabbits, dogs, cats, goats, sheep, pigs and non-human primates.

Animal models may be produced by inserting a selected nucleic acid sequence into a germ line cell or a stem cell using previously described techniques such as oocyte microinjection or transfection or microinjection into embryonic stem cells. Alternatively, an endogenous  $ERR\alpha$  gene may be inactivated or replaced by homologous recombination within embryonic stem cells to produce "knock-out" or "knock-in" animal models. Techniques for obtaining transgenic animals are widely available in the literature.

In accordance with one embodiment of the invention, transgenic animals generated by the introduction of a  $ERR\alpha$  transgene into a fertilized animal oocyte, with subsequent growth of the embryo to birth as a live animal. The  $ERR\alpha$  transgene is a transcription unit which directs the expression of  $ERR\alpha$  gene in eukaryotic cells. To create the transgene,  $ERR\alpha$  gene is ligated with an eukaryotic expression module. The basic eukaryotic expression module contains a promoter element to mediate transcription of  $ERR\alpha$  sequences and signals required for efficient termination and polyadenylation of the transcript. Additional elements of the module may include enhancers which stimulate transcription of  $ERR\alpha$  sequences. The most frequently utilized termination and polyadenylation signals are those derived from SV40 (5). The choice of promoter and enhancer elements to be incorporated into the  $ERR\alpha$  transgene is determined by the cell types in which  $ERR\alpha$  gene is to be expressed. To achieve expression in a broad range of cells, promoter and enhancer elements derived from viruses may be utilized, such as the herpes simplex virus thymidine kinase promoter and polyoma enhancer. To achieve

exclusive expression in a particular cell type, specific promoter and enhancer elements could be used, such as the promoter of the mb-1 gene and the intronic enhancer of the immunoglobulin heavy chain gene. In the present invention, it would be preferred to achieve expression in osteoblasts for example and use a bone specific promoter of bone sialoprotein.

The  $ERR\alpha$  transgene is inserted into a plasmid vector, such as pBR322 for amplification. The entire  $ERR\alpha$  transgene is then released from the plasmid by enzyme digestion, purified and injected into an oocyte. The oocyte is subsequently implanted into a pseudopregnant female animal. Southern blot analysis or other approaches are used to determine the genotype of the founder animals and animals generated in the subsequent backcross and intercross.

Transgenic mice deficient in the production of  $ERR\alpha$  may also be made by homologous recombination. Methods of disrupting the genes of an animal are well established and are described in various publications.

Such deficient mice will provide a model for study of the role of  $ERR\alpha$  in bone cell differentiation and proliferation and general skeletal development. Such animals will also provide tools for screening candidate compounds for their interaction with  $ERR\alpha$  or the signalling pathway activated by  $ERR\alpha$ .

In a further embodiment, the invention provides pharmaceutical compositions comprising  $ERR\alpha$  or a functional analogue or mimetic of  $ERR\alpha$  for the treatment of certain skeletal and connective tissue disorders in which  $ERR\alpha$  expression is abnormal. Such disorders may include but are not limited to osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, periodontal disease, tumoral calcinosis and dermatomyositis. Such compositions as provided herein can be appropriately packaged and targeted to specific cells and/or tissues.

Administration of a therapeutically active amount of a pharmaceutical composition of the present invention means an amount effective, at dosages and for periods of time necessary to achieve the desired result. This may also vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of the  $ERR\alpha$  to elicit a desired response in the subject. Dosage regimens may be adjusted to provide the optimum

therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

By pharmaceutically acceptable carrier as used herein is meant one or more compatible solid or liquid delivery systems. Some examples of pharmaceutically acceptable carriers are sugars, starches, cellulose and its derivatives, powdered tragacanth, malt, gelatin, collagen, talc, stearic acids, magnesium stearate, calcium sulfate, vegetable oils, polyols, agar, alginic acids, pyrogen-free water, isotonic saline, phosphate buffer, and other suitable non-toxic substances used in pharmaceutical formulations. Other excipients such as wetting agents and lubricants, tableting agents, stabilizers, anti-oxidants and preservatives are also contemplated.

The compositions described herein can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable carrier. Suitable carriers and formulations adapted for particular modes of administration are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis the compositions include, albeit not exclusively, solutions of the substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions of the invention may be administered therapeutically by various routes such as by injection or by oral, nasal, buccal, rectal, vaginal, transdermal or ocular routes in a variety of formulations, as is known to those skilled in the art.

The present invention also enables the analysis of factors affecting the expression of the  $ERR\alpha$  gene in humans or in animal models. The invention further provides a system for screening candidate compounds for their ability to turn on or turn off expression of the  $ERR\alpha$  gene and the identification of binding partners which may also affect expression of  $ERR\alpha$  or certain downstream partners.

For example, an ER $\alpha$  cell culture system can be used to identify compounds which activate production of ER $\alpha$  or, once ER $\alpha$  production has been activated in the cells, they can be used to identify compounds which lead to suppression or switching off of ER $\alpha$  production. Alternatively, such a cell culture system can be used to identify compounds or binding partners of ER $\alpha$  which increase its expression. Compounds thus identified are useful as therapeutics in conditions where ER $\alpha$  production is deficient or excessive.

The present invention enables also a screening method for compounds of therapeutic utility as antagonists of the biological activity of ER $\alpha$ . Such antagonist compounds are useful, for example, to reduce or prevent differentiation and maturation of osteoblasts and osteocytes. ER $\alpha$  antagonists may also be used in the treatment of bone related disorders involved in inappropriate bone cell growth. Those skilled in the art will be able to devise a number of possible screening methods for screening candidate compounds for ER $\alpha$  antagonism.

A screening method may also be based on binding to the ER $\alpha$  receptor. Such competitive binding assays are well known to those skilled in the art. Once binding has been established for a particular compound, a biological activity assay is employed to determine agonist or antagonist potential.

The present invention further enables therapeutic intervention in disorders associated with an inappropriate level or location of ER $\alpha$  expression.

Such interventions include;

(a) in conditions associated with undesired biological activity of ER $\alpha$ , inhibition of its activity by administration of antagonist compounds, anti-ER $\alpha$  antibodies, agents to down-regulate gene expression or alternatively antisense methods to inhibit gene function. ER $\alpha$  can be conjugated with selected molecules to target specific tissues inappropriately over producing ER $\alpha$ .



## **Examples**

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of chemistry, molecular biology, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

### **ERR $\alpha$ is differentially expressed as osteoprogenitor cells differentiate to mature osteoblasts and osteocytes**

ERR $\alpha$  mRNA expression levels were assessed over a proliferation-differentiation time course by Northern blotting of primary rat calvaria (RC) cells grown in the presence (+Dex) or absence (-Dex) of dexamethasone (Dex). In cultures grown without Dex, in which few nodules formed, ERR $\alpha$  mRNA was expressed in proliferating RC cells (day 6), but decreased to lower levels in cells undergoing condensation and early nodule formation (day 10) and nodule mineralization (day 15) (Fig. 1). In cultures treated with Dex, in which nodule formation was increased significantly, ERR $\alpha$  levels were similar to those seen in -Dex cultures at all time points (Fig. 1). For comparison, mRNA levels for two osteoblast markers, alkaline phosphatase, a relatively early marker of osteoblast development, and osteocalcin, a marker of osteoblast maturation, are also shown.

Because RC cell cultures comprise a heterogeneous mixture of cell types and osteoblasts at different differentiation stages, it was sought to confirm that ERR $\alpha$  is expressed by osteoblast lineage cells. Globally-amplified (poly(A) PCR) cDNA pools prepared previously from single isolated osteoblast colonies at different stages of differentiation were used to do this. Colonies used were selected based on their molecular phenotypes (expression of osteopontin, bone sialoprotein, alkaline phosphatase and osteocalcin). ERR $\alpha$  was amplified in each cDNA pool with specific primers for sequences in the 3' UTR of ERR $\alpha$ . ERR $\alpha$  was found to be expressed at all developmental times

(Fig. 2). Notably, mRNA levels were generally lower in primitive progenitors (A), more mature precursors (B), and osteoblastic cells associated with mineralized nodules (E), and higher in preosteoblasts (C) and osteoblasts (D) (Fig. 2).

**ERR $\alpha$  mRNA is more highly expressed in RC cell cultures than either ER $\alpha$  and ER $\beta$  mRNA and expression patterns vary**

The level of ERR $\alpha$  expression was compared with those of the two estrogen receptors, ER $\alpha$  and ER $\beta$ . RT-PCR was done with primers specific for each of these three receptors. ERR $\alpha$  was found to be expressed at significantly higher levels than either ER $\alpha$  and ER $\beta$  and the two estrogen receptors were themselves present at different levels (i.e., ERR $\alpha$  was easily detected at 25 cycles, while 35 cycles and 40 cycles were required to detect ER $\alpha$  and ER $\beta$  respectively). In addition, the expression patterns of the three receptors over the proliferation-differentiation time course in RC cell cultures was strikingly different. Similarly to ERR $\alpha$ , ER $\beta$  decreased over time in -Dex cultures, whereas ER $\alpha$  increased (Fig. 3). On the other hand, both ER $\alpha$  and ER $\beta$  decreased over time in +Dex cultures in a manner similar to ERR $\alpha$  (cf. Fig. 1 and 3).

**Cell culture**

For cell cultures, cells were enzymatically isolated from the calvaria of 21 d Wistar rat fetuses by sequential digestion with collagenase as described previously (Bellows, 1986). Cells obtained from the last four of the five digestion steps (populations II-V) were pooled and plated in T-75 flasks in  $\alpha$ -MEM containing 15% heat-inactivated FBS (Flow Laboratories, McLean, VA) and antibiotics comprising 100 mg/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 50 mg/ml gentamycin (Sigma), and 0.3 mg/ml fungizone (Flow Laboratories). After 24hrs incubation, attached cells were washed with PBS to remove nonviable cells and other debris, and then collected by trypsinization using 0.01% trypsin in citrate saline. Aliquots were counted with a Coulter Counter, and the remaining

cells were resuspended in the standard medium described above. The resuspended cells were plated into 100 mm tissue culture dishes at  $10^5$  cells, into 35mm tissue culture dishes at  $2 \times 10^4$  and in 24 wells plates at  $10^4$ . After 24hrs incubation, medium was changed and supplemented with 50 mg/ml ascorbic acid, 10 mM sodium  $\beta$ -glycerophosphate, and with or without  $10^{-8}$  M dexamethasone,  $10^{-8}$  M of E2 (Merck, Sharp, and Dohme, Canada, Ltd., Kirkland, PQ) or  $10^{-8}$  M of E2 (Sigma), or  $10^{-9}$  M of 1,25Vit D3. Medium was changed every 2 days. All dishes were incubated at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub> incubator.

#### Northern blots

Total RNA was extracted with guanidine from RC cells at different times of the culture corresponding to different stages of proliferation, differentiation and bone nodule formation (Current Protocols in Molecular Biology, vol. 1, 1996). Northern blots were prepared and hybridized with a 750bp fragment corresponding to the rat 3' UTR of ERR $\alpha$  (J.M. Vanacker) according to standard procedures (Chirgwin et al, 1979). Rat bone/liver/kidney ALP (Noda et al., 1987; gift of Dr. G.A. Rodan, Merck Sharpe, and Dohme Research Laboratories, West Point, PA) was a 600 bp cDNA *EcoRI* fragment obtained by digesting pRAP54 with *BssHII-XhoI* to remove 1.8 kb of 5' region and religating the blunt ends. Rat OPN (Dr. R. Mukherjee, Montreal, PQ) was a 700 bp cDNA *BamHI-EcoRI* fragment obtained by digesting full length cDNA with *PvuI* to remove 800 bp of 5' region and ligating the blunt ended fragment into *SmaI* cut pGEM-7Zf(+) vector (Promega, Madison, WI). Rat OCN was a partial cDNA containing 350 bp of the 3' UTR isolated with OCN-specific primers from a  $\lambda$ gt11 library prepared from ROS 17/2.8 cells. Identity was confirmed by sequencing (Liu et al., 1994).

## RT-PCR

Samples of total cellular RNA (5 µg) were reverse-transcribed using oligo dT and the first strand synthesis kit of Superscript<sup>TM</sup> II. Gibco BRL. PCR was performed with primers specific for ERR $\alpha$ , ER $\alpha$  and ER $\beta$ . Primers, located in different exons, were as follows:

ERR $\alpha$  upstream (3'UTR) CAG GAA AGT GAA TGC CCA GG

ERR $\alpha$  downstream (3'UTR) CTT TGC AGC AAA TAT ACA TT

ER $\alpha$  upstream (Dom D 5') GAG CTG CCA ACC TTT GGC CAA GT

ER $\alpha$  downstream (Dom D 3') TGA ACT TGA TCG TGG AGA TTC

ER $\beta$  upstream ( Dom D) AAA GCC AAG AGA AAC GGT GGG CAT

ER $\beta$  downstream (Dom E) GCC AAT CAT GTG CAC CAG TTC CTT

L32 upstream CAT GGC TGC CCT TCG GCC TC

L32 downstream CAT TCT CTT CGC TGC GTA GCC

The PCR reaction mixture contained cDNA (1 µl), 1 µl dNTP mix (20mM), 10x PCR buffer, 25 pmol primers and 5 Units of Taq polymerase from Quiagen. PCR was done for 25 cycles (94°C for 1 min, 55°C for 1min, 72°C for 1min and a final elongation step of 7 min at 72°C) for ERR $\alpha$  and L32; 35 cycles (94°C for 1 min, 55°C for 1min, 72°C for 1min and a final elongation step of 7 min at 72°C) for ER $\alpha$ ; 45 cycles (94°C for 1 min, 59°C for 1min, 72°C for 1min and a final elongation step of 7 min at 72°C) for ER $\beta$ . Amplimers were sequenced for verification.

## Poly (A) PCR Library selection

Twenty poly(A) PCR libraries representative of five transitional stages in osteoblast lineage progression were selected from more than one hundred available amplified colonies (Liu, F and Aubin, J.E., 1998). Stage A are replica-plated monolayer colonies committed to differentiate to the osteoblast lineage but not yet expressing type I

αI collagen or alkaline phosphatase, both early markers of osteoprogenitor cells. Stage B and C colonies are progressively more mature, i.e. expressing type I αI collagen or both type I αI collagen and alkaline phosphatase respectively. Stage D colonies represent multilayered cells and contain histologically identifiable cuboidal osteoblasts. Stage E colonies comprised terminal differentiation stages, with multilayered cells and mineralized bone matrix. Relative amounts of total cDNA were determined by Southern hybridization and were used for normalization.

#### Western blots

Total protein was extracted from confluent HeLa cells according to standard methods (Current Protocols in Molecular Biology, vol. 1, 1996) and Western blot analyses were performed using a semi-dry system. Immunoblotting was performed with rabbit antiserum prepared against a rat peptide (NH-CPASDECEITKRR-C) localized in the C domain of ERRα (J.M. Vanacker). Blots were incubated overnight at room temperature with the antiserum diluted to 1/500, and binding was detected using HRP-conjugated goat-anti-rabbit antibodies (Biorad).

#### **ERRα, ERα and ERβ protein is also expressed in RC cultures, but only ERRα and ERα are detectable in bone nodules**

To determine whether and when in RC cultures the three receptors were expressed, immunocytochemistry was performed using polyclonal antibodies specific for ERα and ERβ (Santa Cruz) and antiserum prepared against ERRα in +Dex-treated RC cultures. To confirm the specificity of the ERRα antiserum, a Western blot on Hela cells was done. As expected based on previously published data (Johnston et al, 1997, Shigeta et al, 1997), a single immunoreactive band at 53Kd in Hela cells (Fig 4R) was detected. Interestingly, ERα was detected in RC cells at all times analysed from early proliferation stages through nodule formation and mineralization (Fig. 4 B, E, H, K, N, Q); note

especially the strong labeling of osteoblastic cells in nodules (Fig. 4N, Q). ER $\beta$ , on the other hand, was more difficult to detect at any time other than in early proliferating cultures (Fig. 4C, E, I, L, O), in particular, ER $\beta$  in osteoblastic cells in bone nodules (Fig. 4O) was seldom detected.

As predicted based on the relatively wide tissue distribution of ERR $\alpha$  mRNA (Bonnelye et al., 1997b), ERR $\alpha$  protein was found more widely distributed in RC cell cultures than either ER $\alpha$  or ER $\beta$ . ERR $\alpha$  was found in most if not all cells in RC cell cultures from early proliferation stages through mineralized nodule formation (Fig. 4A, D, G, J, M, P); note especially, however, that staining for ERR $\alpha$  was more intense in the osteoblasts associated with both early and late bone nodules than in the surrounding fibroblastic cells (Fig. 4J, M). Interestingly, however, while ERR $\alpha$  is primarily found in the cytoplasm and perinuclear location at day 2 and day 4 (Fig. 4A, D), by day 6 and thereafter, including in mature osteoblasts, it is primarily localized in the nucleus (Fig. 4G, J, M, P). ER $\alpha$  was mainly nuclear from day 2 (Fig. 4B) to day 6 but thereafter was cytoplasmic and nuclear (Fig. 4N, Q), while ER $\beta$  was primarily perinuclear in all cells in which it could be detected (e.g., Fig. 4F).

#### **ERR $\alpha$ is more highly and widely expressed *in vivo* in fetal rat calvaria compared ER $\alpha$ and ER $\beta$**

To extend the observations made *in vitro* to bones *in vivo*, immunocytochemistry was performed on 21d. fetal rat calvaria sections. Consistent with the *in vitro* results, strikingly different expression patterns were seen for ERR $\alpha$ , ER $\alpha$  and ER $\beta$ . ERR $\alpha$  was found in all detectable cohorts of osteoblasts from those associated with nascent bone at the osteogenic front (Fig. 5A) to those in more mature bone trabeculae (Fig. 5B) including spongy bone (Fig. 5C). Consistent with our RT-PCR results on single bone nodules (Fig. 2), ERR $\alpha$  was also detectable in sutural cells (Fig. 5A), preosteoblasts (Fig. 5C) and osteocytes (Fig. 5B). ER $\alpha$ , on the other hand, was not detected in any cells in the suture

or osteogenic front (Fig. 5D), but was detected in some osteoblasts associated with more mature (Fig. 5E) and spongy bone (Fig. 5F). ER $\beta$  was detected in a pattern virtually reciprocal to that of ER $\alpha$ , i.e., it was present in sutural cells and cells at the osteogenic front (Fig. 5G), but it was virtually undetectable in osteoblastic cells in more mature and spongy bone (Fig. 5H, I). Based on staining intensity, and in keeping with our RT-PCR results, ERR $\alpha$  was more highly expressed than either ER $\alpha$  and ER $\beta$  *in vivo*.

#### Immunolabelling

Immunolabelling of cultures was done essentially as described previously (Turksen, 1991; Turksen, 1992). Cultures were rinsed with PBS, fixed with 3.7% formaldehyde in PBS and permeabilized with methanol at -20°C. Frozen sections were fixed 10 min in cold acetone. After rinsing, cells in dishes or frozen sections were incubated for 1 hr at room temperature with 10% normal serum in PBS for ERR $\alpha$  and ER $\alpha$  and in 3% BSA in PBS (denaturated) for ER $\beta$ . After rinsing, cells or sections were incubated for 1.5 hours with appropriate dilutions of primary antibodies (1/500, anti-ERR $\alpha$ , as above; anti-ER $\alpha$  or anti-ER $\beta$  (MC-20 or Y-19, respectively, Santa Cruz Biotechnology, Inc)). 10% normal serum in PBS or 3% BSA in PBS were used as negative controls. Nodules or calvaria sections were rinsed in PBS and incubated for 1 hr at room temperature with CY-3-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Lab, West Grove, PA, USA; 1/300 final dilution) for ERR $\alpha$  and ER $\alpha$  and with an anti-goat antibody for ER $\beta$ . After rinsing, samples were mounted in Moviol (Hoechst Ltd, Montreal, PQ, Canada) and observed by epifluorescence microscopy on a Zeiss Photomicroscope III (Zeiss, Oberkochen, Germany). For photography and printing, equal exposure times were used for specifically-labelled and control cultures.

### In Situ Hybridization

Frozen sections for *in situ* hybridization were fixed for 20 minutes in 4% paraformaldehyde, dehydrated and stored until used at -70°C. The hybridization protocol was as described previously (Zellar and Rogers, 1989), except that sections were not treated with pronase so as to preserve the cellular morphology. Briefly, sections were acetylated and RNA probes in hybridization mix (0.48M NaCl, 8mM Tris-Cl, pH 7.5, 1.6 mM EDTA, 0.8x Denhardt's solution, 0.4 mg/ml yeast tRNA, 80mg/ml poly(A), 40% deionized formamide, 20% dextran sulfate) were applied for hybridization for 4 hours at 42°C. After hybridization, sections were washed at 50°C twice with wash solution I (2x SSC, 50% Formamide, 0.1%  $\beta$ -mercaptoethanol), treated with Rnase A (20mg/ml boiled Rnase A, 0.5 M NaCl, 10mM Tris-Cl) for 1 hour at 37°C and washed with wash solution II (0.1x SSC, 1%  $\beta$ -mercaptoethanol) as described (Zellar and Rogers, 1989). The <sup>35</sup>UTP-labelled RNA probe used was 750bp fragment of the 3'UTR of rat ERR $\alpha$ .

### Nodule quantification

For quantification of nodule formation, dishes or wells were fixed and stained by the Von Kossa technique and bone nodules were counted on a grid (Bellows et al., 1986, Bellows and Aubin, 1989). Results are plotted as the mean number of nodules  $\pm$  SD of three dishes for controls and each concentration of antisense or sense primers.

### Cell counting

For cell growth analysis, the cell layers were rinsed in PBS, released with trypsin and collagenase (1:1, vol/vol, of solutions described above), and the harvested cells were counted electronically. Results are plotted as the average of three counts for each of two dishes for each concentration of antisense or sense primers used.



### Alkaline phosphatase histochemistry

The histochemical stain for alkaline phosphatase is a modification of Pearse's (1960). Cells were rinsed once with cold PBS and fixed in 10% cold neutral buffered formalin for 15 min, rinsed with distilled water, and left in distilled water for 15 min. Fresh substrate (10 mg Naphthol AS MX-PO<sub>4</sub> (Sigma) dissolved in 400  $\mu$ l N,N-dimethylformamide, then added to 50 ml distilled water and 50 ml Tris-HCl (0.2 M, pH 8.3) and then 60 mg Red Violet LB salt (Sigma)), was filtered through Whatman's No. 1 filter directly onto the dishes, and incubated for 45 min at 20°C. The dishes were then rinsed in tap water, drained and stained with 2.5% silver nitrate for 30 min at room temperature (von Kossa stain). After rinsing twice in distilled water, toluidine blue was applied for 2 seconds and the dishes were then rinsed 3 times with tap water. The dishes were finally air dried.

### Transient transfections

Primary RC cells were grown in 35mm tissue culture dishes at  $2 \times 10^4$ /dish in  $\alpha$ -MEM containing 10% heat-inactivated FBS (Flow Laboratories, McLean, VA) and supplemented with 50 mg/ml ascorbic acid, 10 mM sodium  $\beta$ -glycerophosphate, and  $10^{-8}$  M dexamethasone. Cells were transfected at 50% of confluence according to the Effecten transfection protocol (Quiagen) using a pcDNA empty plasmid as a control and pcDNA3-ERR $\alpha$  (in the EcoRI cloning site) at 0.5 to 1 $\mu$ g of total DNA per transfection. As control of transfection efficiency, a CMV-  $\beta$ Gal vector was used. Nodules were counted at 15 days.

### ERR $\alpha$ is required for proliferation of RC cells and for the formation of bone nodules

Given its expression in both proliferating osteoprogenitor cells and more mature osteoblasts and osteocytes, it was next determined whether ERR $\alpha$  is a critical factor in osteoblast proliferation or differentiation. Antisense oligonucleotides form DNA:RNA

duplexes with specific mRNA species, thereby blocking binding of the mRNA to the 40S ribosomal subunit and preventing translation (Reddy et al. 1994). An antisense oligonucleotide specific for  $ERR\alpha$  was used and found a dose dependent decrease in cell number at day 2 (Fig. 6). These results suggest that  $ERR\alpha$  plays a role in the proliferation of RC cells, an effect that may also influence bone nodule number later in treated cultures.

To determine whether  $ERR\alpha$  may play a role in osteoblast differentiation, independently of an effect on proliferation, RC cells were treated with the antisense oligonucleotide beginning at day 6 (after cells had reached confluence). While sense oligonucleotides had a small dose dependent effect on nodule numbers, antisense oligonucleotides caused a striking dose dependent decrease in bone nodule formation, i.e., 70% at 0.5  $\mu$ M, and 100% at 1  $\mu$ M and 2  $\mu$ M (Fig. 7). On the other hand, overexpression of  $ERR\alpha$  with transient transfection of RC cells, resulted in a dose dependent biphasic effect: transfection with 0.5  $\mu$ g of  $ERR\alpha$ -containing vector increased (~ 20%) the number of mineralized bone nodules whereas transfection with 0.5  $\mu$ g of  $ERR\alpha$ -containing vector decreased (40%) the number (Fig. 8).

#### **$ERR\alpha$ expression is stimulated by estrogen and vitamin D3 in proliferating RC cells cultures**

To determine whether other hormones that influence proliferation and differentiation in RC cell cultures may modulate  $ERR\alpha$  levels, RC cells were treated continuously with estrogen ( $E_2$ ,  $10^{-8}$ M) or 1,25(OH) $_2$  vitamin D $_3$  (D $_3$ ,  $10^{-9}$ M). Both  $E_2$  (40% increase) and D $_3$  (47% increase) stimulated expression of  $ERR\alpha$  mRNA at day 6, but not later (Fig. 9). An acute exposure of RC cells to either  $E_2$  or D $_3$  for 24 hours at beginning from day 9 (nascent nodule formation) or day 15 (mature nodules present) had any effect on  $ERR\alpha$  mRNA levels (Fig. 9).

Antisense and sense oligonucleotide treatment

The resuspended cells were plated /in 24 wells plates at  $10^4$ /dish. Antisense oligonucleotide inhibition of  $ERR\alpha$  expression was accomplished with a 20-base phosphorothioate-modified oligonucleotide, localized to the A/B domain. The  $ERR\alpha$  antisense oligonucleotide sequence was 5'-TCACCGGGGGTTCAGTCTCA-3'. Control dishes were treated with the complementary sense oligonucleotide or no oligonucleotide. Preliminary experiments were done to determine effective oligonucleotide concentrations that were not toxic. 0.1  $\mu$ M to 5  $\mu$ M oligonucleotides were added directly to cells either during the proliferation phase (days 1 to 6) and 0.1  $\mu$ M to 2  $\mu$ M oligonucleotides were added for the differentiation phase (days 6 to 12) in standard medium as above supplemented with 50 mg/ml ascorbic acid, 10 mM sodium  $\beta$ -glycerophosphate, and  $10^{-8}$  M dexamethasone. Medium was changed every 2 days and fresh oligonucleotides were added.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described.

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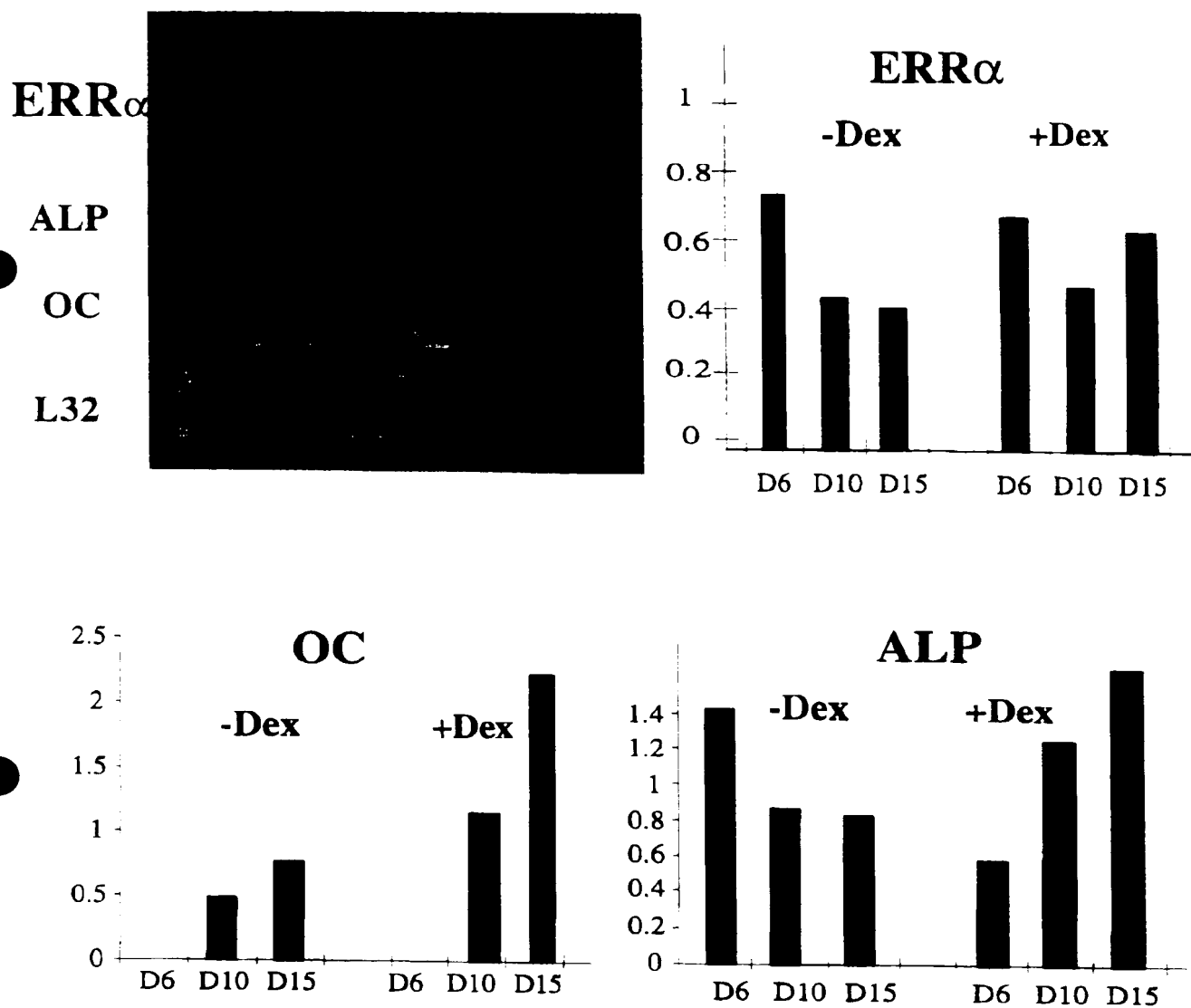
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**Figure 1**

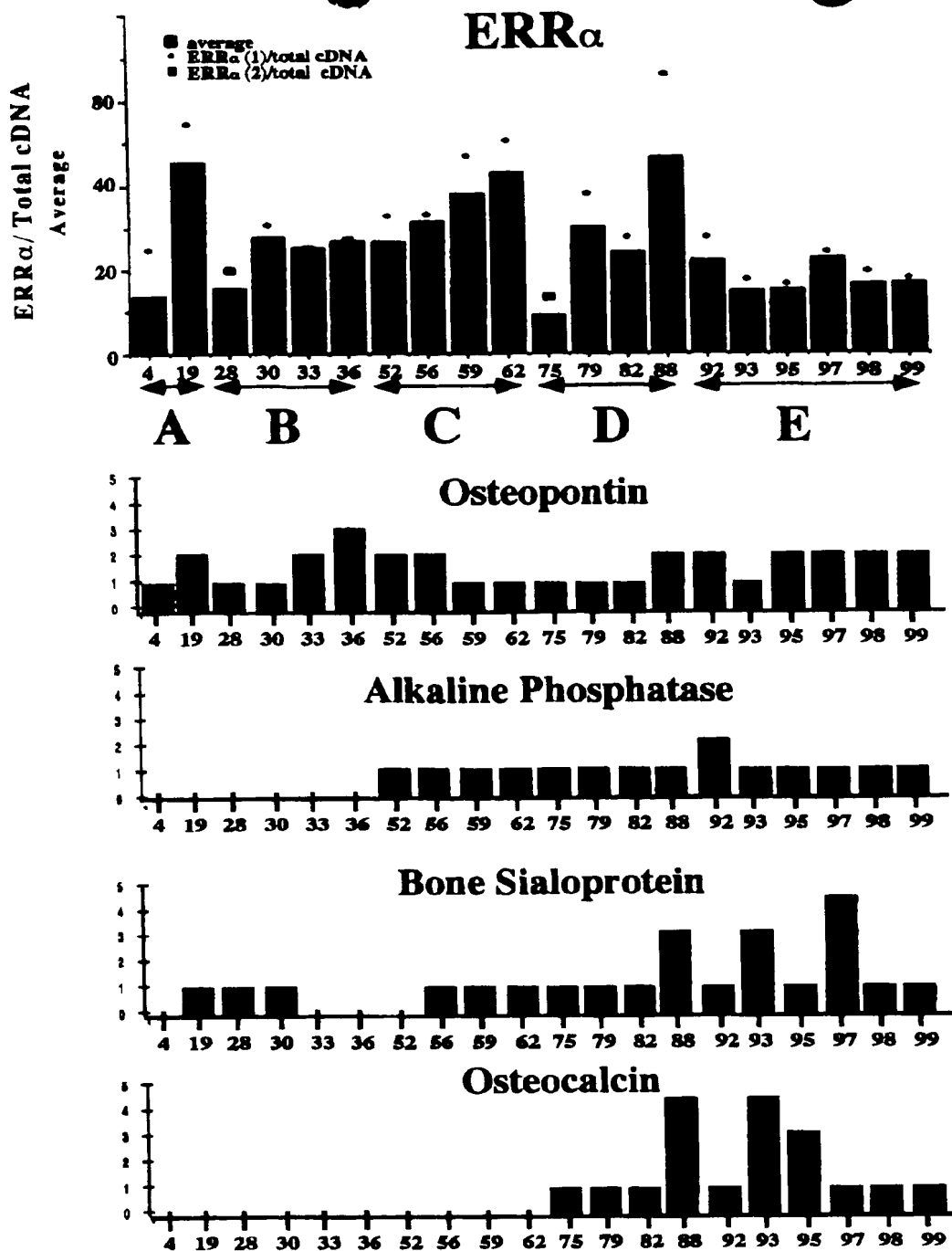
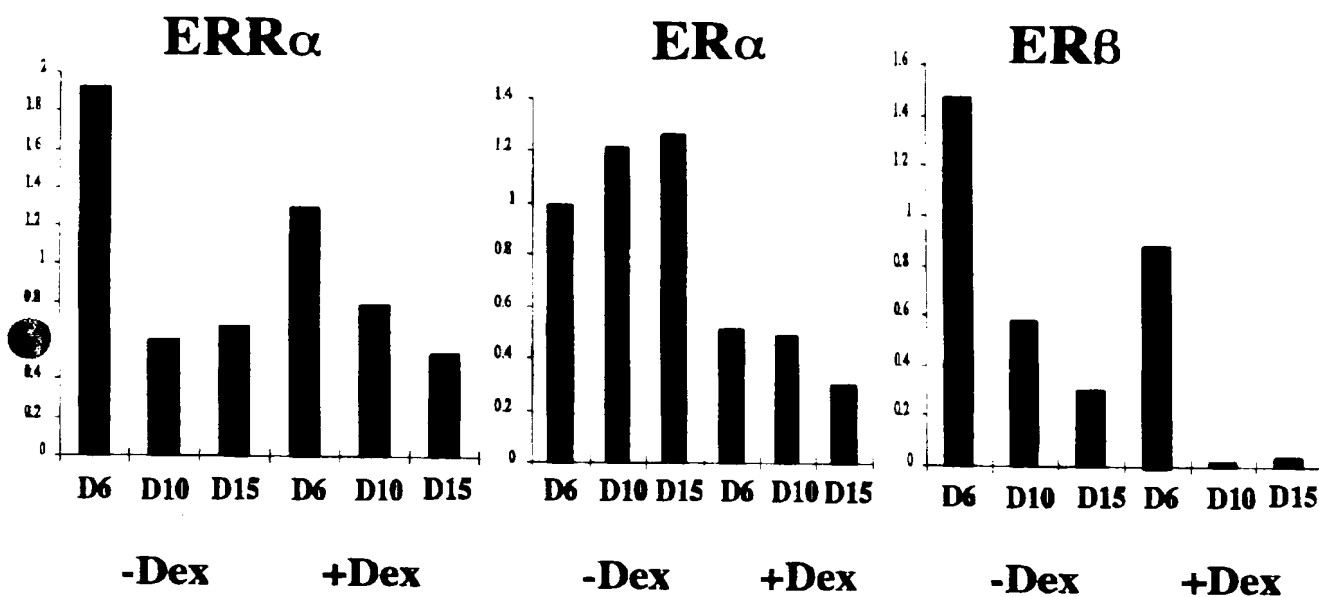
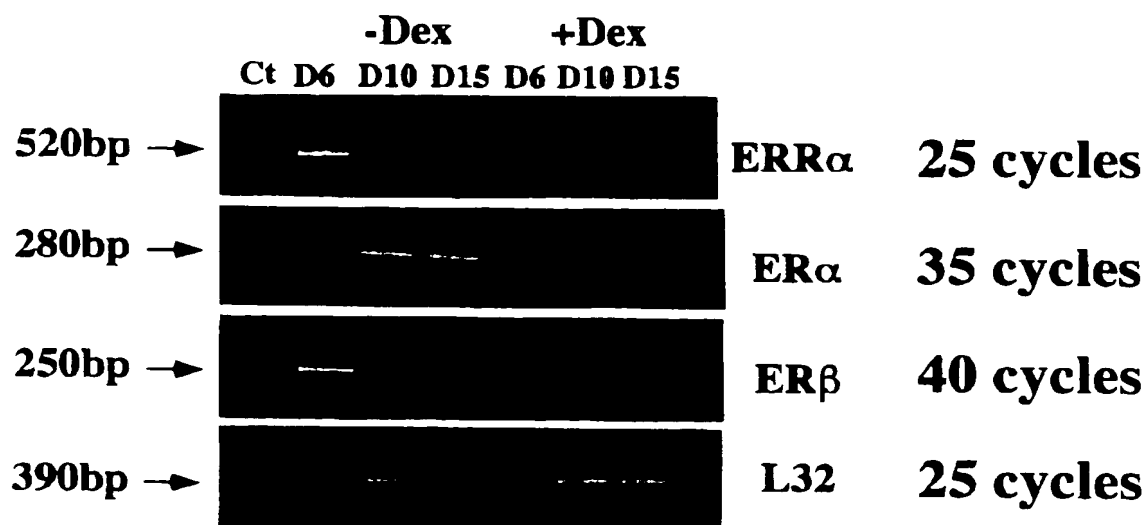
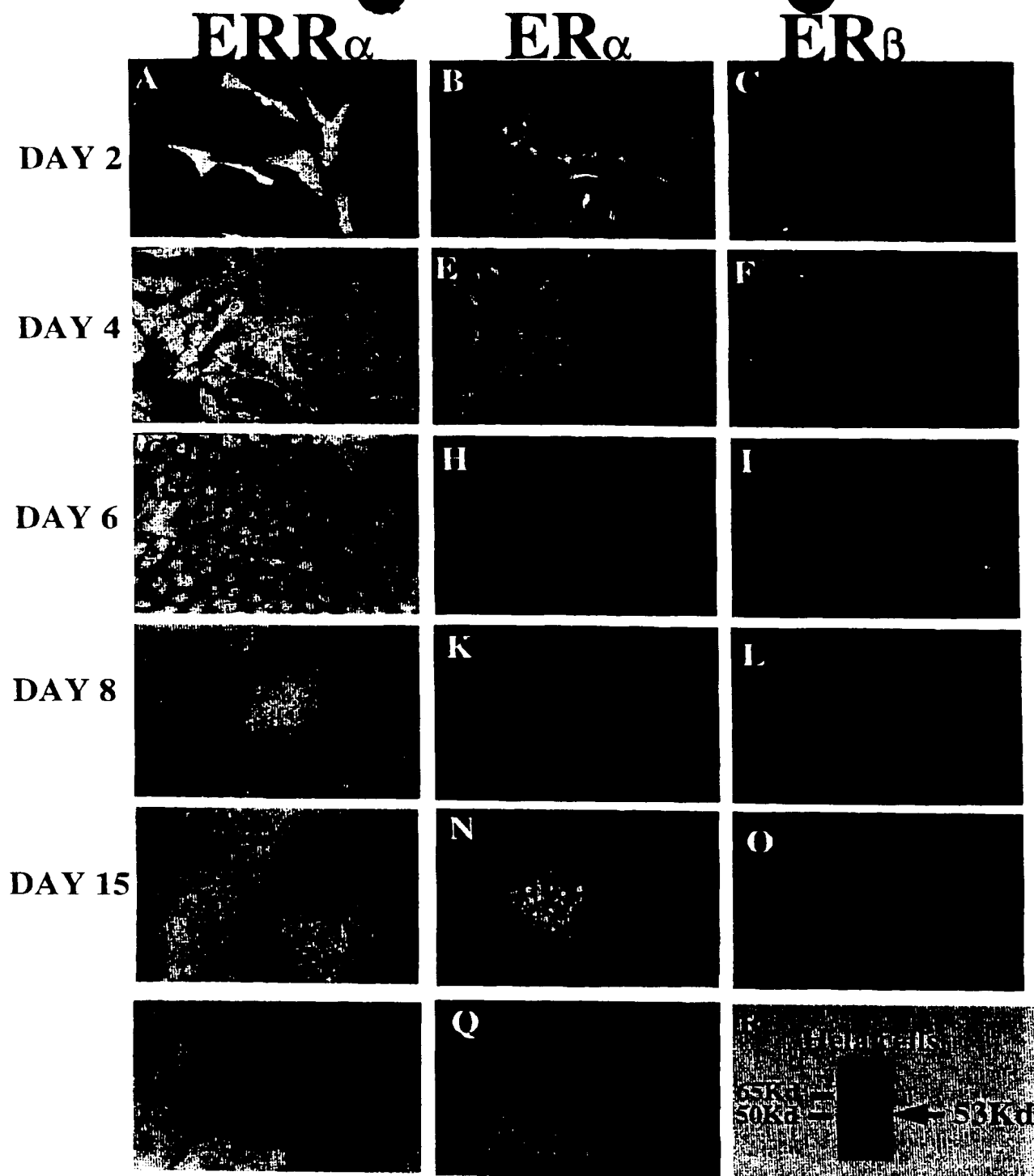


Figure 2

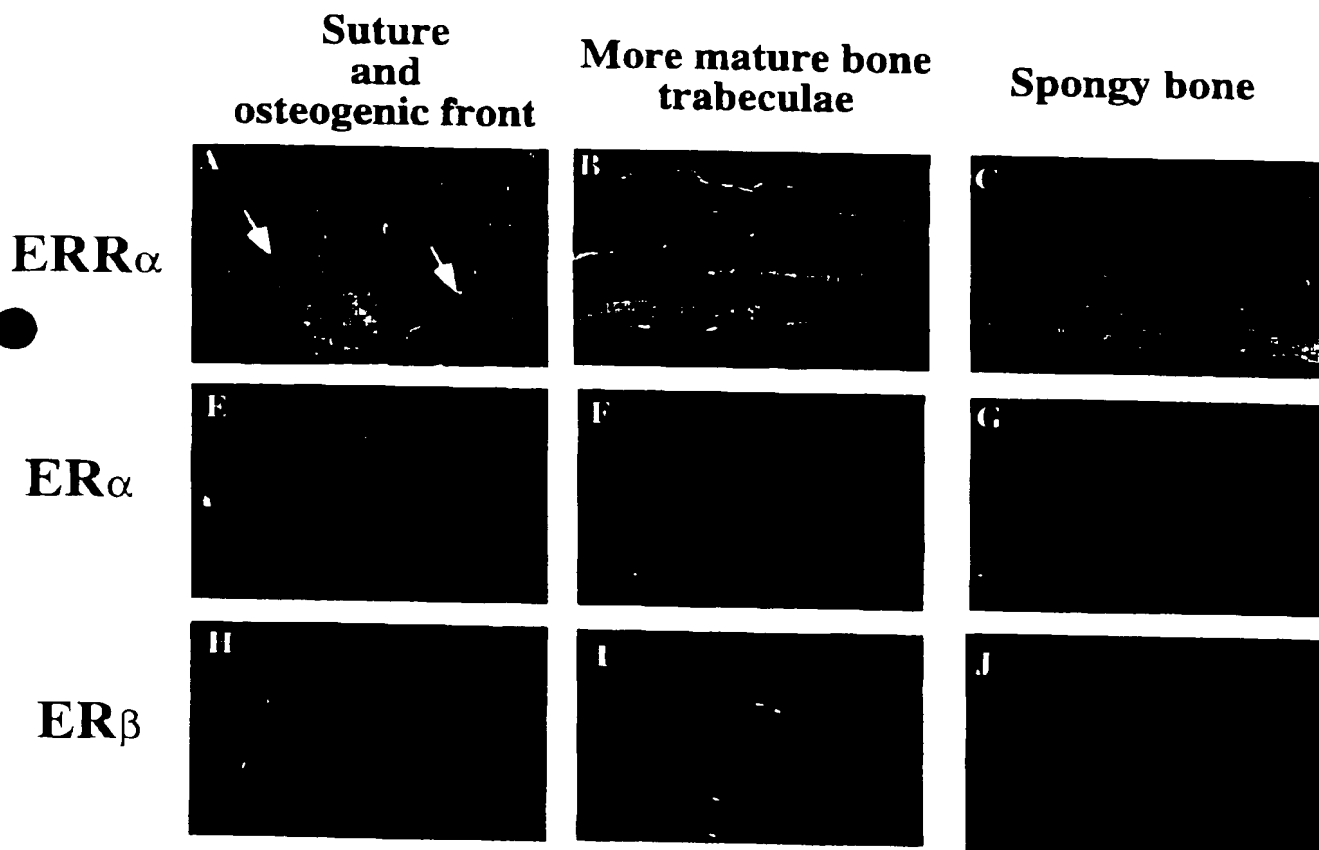


**Figure 3**





**Figure 4**



**Figure 5**

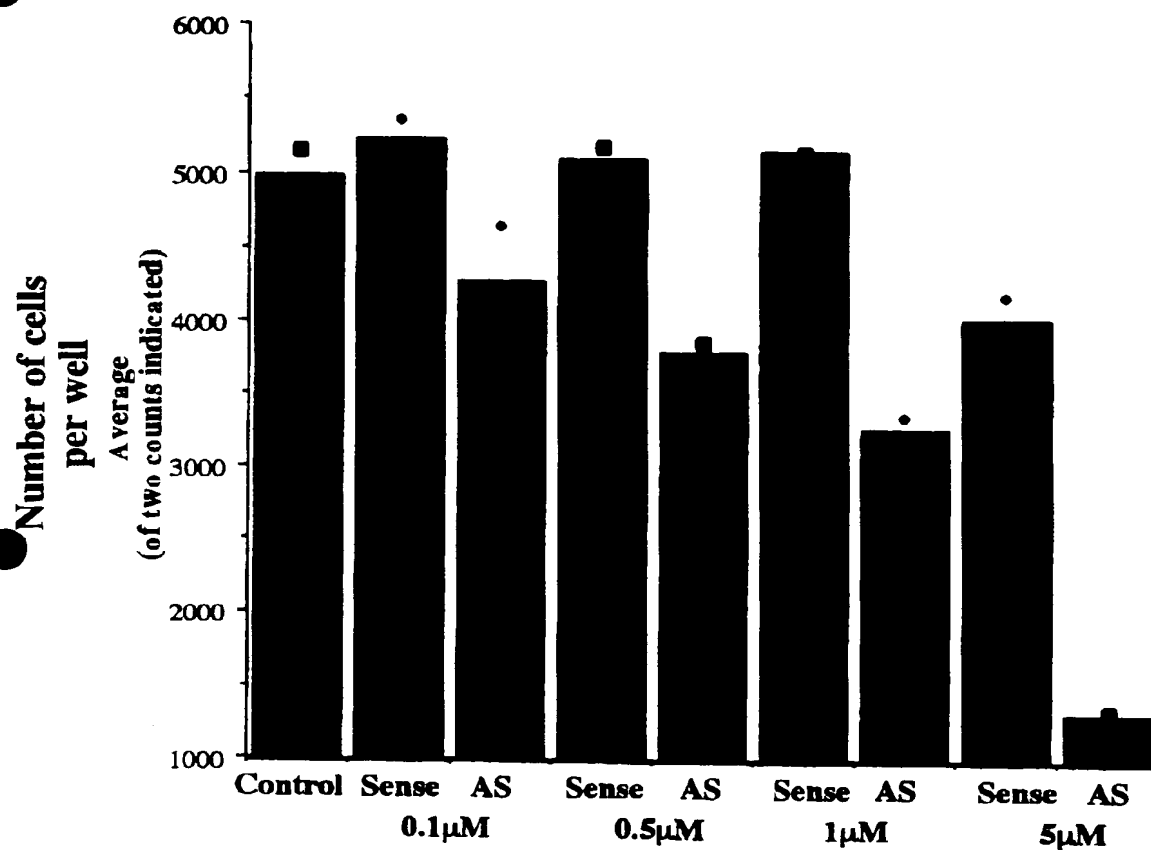
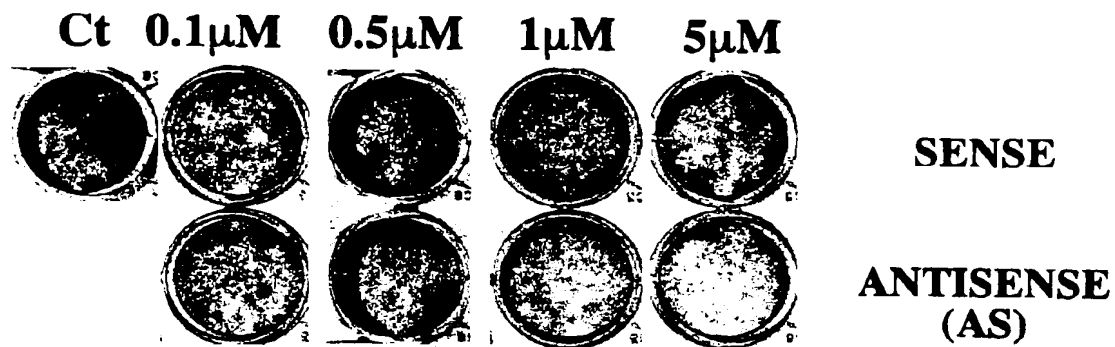


Figure 6

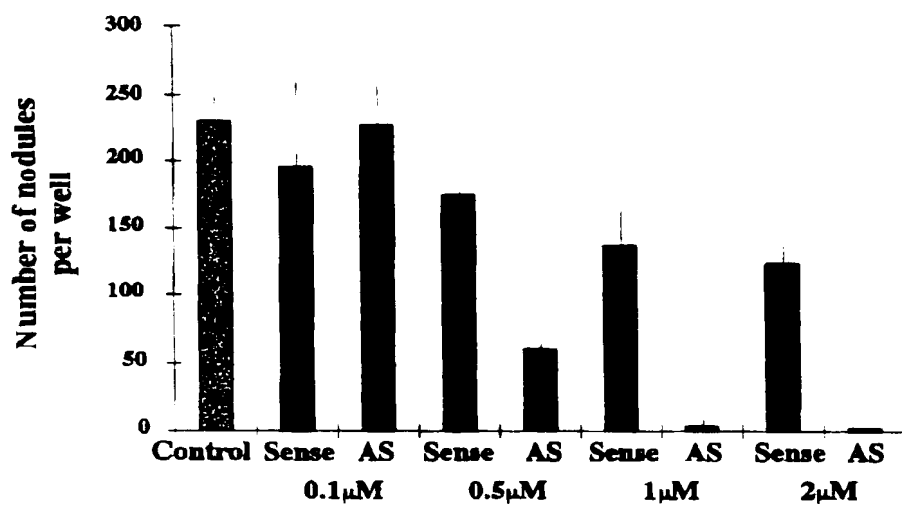
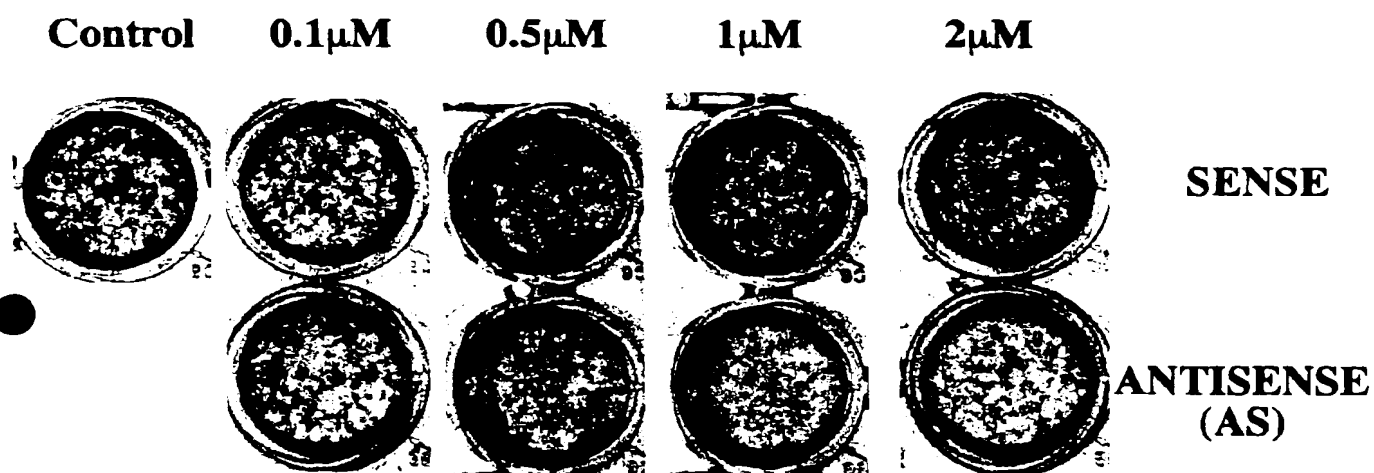
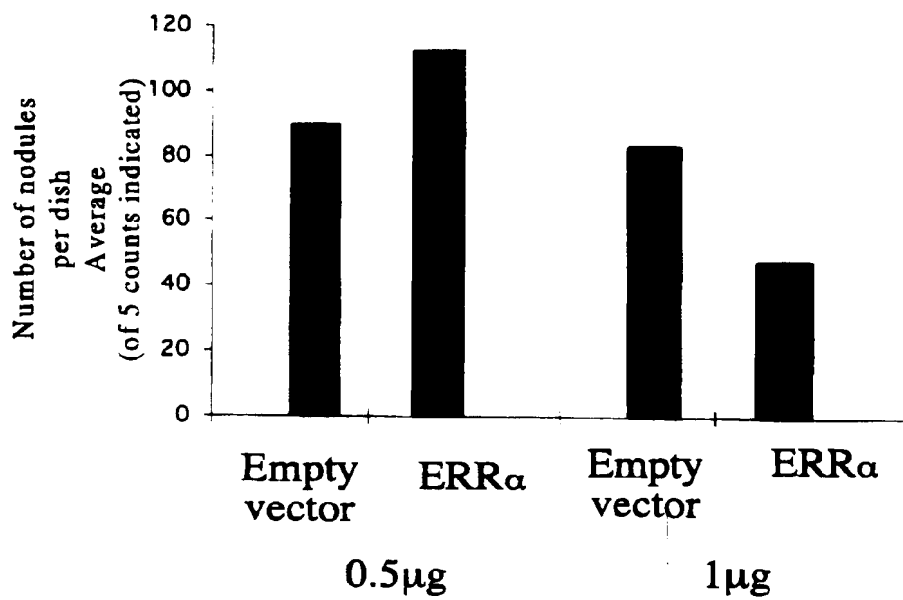
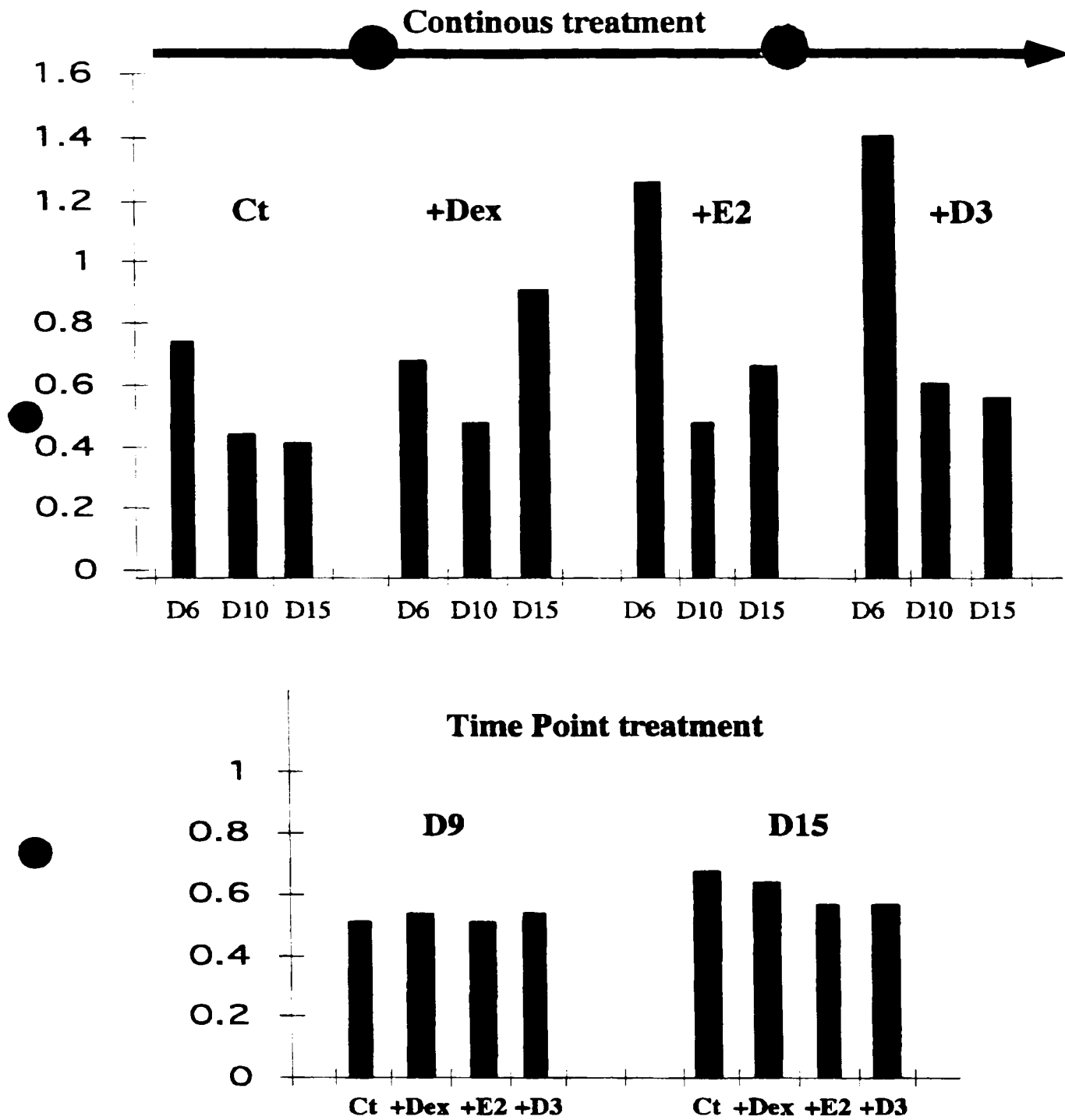


Figure 7

**Figure 8**

**Figure 9**

